Development of Novel Lipophilic Derivatives of DADLE (Leucine Enkephalin Analogue): Intestinal Permeability Characteristics of DADLE Derivatives in Rats

Tomomi Uchiyama,[1] Atsushi Kotani,[1] Hiroyuki Tatsumi,[1] Takeshi Kishida,[1] Aya Okamoto,[1] Naoki Okada,[1] Masahiro Murakami,[1] Takuya Fujita,[1] Youichi Fujiwara,[2] Yoshiaki Kiso,[2] Shozo Muranishi,[1] and Akira Yamamoto[1,3]

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Purpose: The objective of this study is to examine the intestinal permeability of novel lipophilic derivatives of DADLE (Tyr-D-Ala-Gly-Phe-D-Leu), an enkephalin analogue, using isolated rat intestinal membranes.

Methods: The novel lipophilic derivatives of DADLE were synthesized by chemical modification with various fatty acids at the C terminus. The pharmacological activities of these DADLE derivatives were assessed by a hot plate test. The intestinal permeability of these derivatives was estimated by the *in vitro* Ussing chamber method.

Results: We obtained four different DADLE derivatives including acetyl-DADLE (DADLE-C2), butyryl-DADLE (DADLE-C4), caproyl-DADLE (DADLE-C6), and caprylyl-DADLE (DADLE-C8). All the derivatives of DADLE had at least 75 % of the activity of native DADLE, suggesting that chemical modification of DADLE at the C terminus did not markedly affect its pharmacological activity. These DADLE derivatives were more stable than native DADLE in jejunal and colonic homogenates. A "bell-shaped" profile was observed between the apparent permeability coefficients (Papp) of DADLE derivatives and lipophilicity. In particular, DADLE-C4 had the greatest permeability characteristics across the intestinal membrane of the acyl derivatives studied in this experiment. The permeability of DADLE-C4 across the jejunal membrane was further improved in the presence of puromycin, amastatin, and sodium glycocholate (NaGC), all at a concentration of 0.5 mM.

Conclusions: We suggest that the combination of chemical modification with butyric acid and the application of a protease inhibitor are effective for improving the absorption of DADLE across the intestinal membrane.

KEY WORDS: drug absorption; peptide delivery; enkephalin; absorption enhancement; chemical modification; protease inhibitor.

INTRODUCTION

The bioavailability of peptide and protein drugs after oral administration is generally poor because they are extensively degraded by proteases in the gastrointestinal tract, and they are poorly absorbed from the gastrointestinal tract (1,2). Therefore, various strategies, such as the use of absorption enhancers and protease inhibitors, have been utilized to improve the permeability of these peptides from the gastrointestinal tract (1,2). However, some of these additives may cause local irritation of the intestinal mucosa and nonselective absorption of other exogenous compounds. Therefore, alternative methods are required to enhance the selective absorption of peptide and protein drugs from the gastrointestinal tract.

Chemical modification of peptides is a potentially useful approach because this method can alter the various physicochemical properties of peptides, such as increasing lipophilicity and permeability across the intestinal membrane (3). Furthermore, it has been reported that chemical modification of peptides can increase resistance to enzymatic degradation (3). Based on these findings, we synthesized novel lipophilic derivatives of insulin (4–6), calcitonin (7), tetragastrin (8–11), thyrotropin releasing hormone (TRH) (12,13), and phenylalanyl-glycine (Phe-Gly) (14) by covalent attachment of various saturated fatty acids. We found that acylation improved the intestinal absorption of these peptide and protein drugs (4–14).

Leucine-enkephalin (Leu-Enk) and methionine-enkephalin (Met-Enk) are naturally occurring analgesic pentapeptides which are known to act as neurotransmitters or neuromodulators in pain transmission. Their analgesic activities are, however, rather short in duration. This is due to their rapid inactivation by enzymes in various organs, including the gastrointestinal tract (15). In addition, the intestinal absorption of enkephalins is known to be very poor due to their extensive degradation in the gut (16). Consequently, various protease inhibitors have been used to improve the stability and permeability of enkephalins (17,18). We reported that protease inhibitors such as amastatin, puromycin, bacitracin, and NaGC improved the stability and permeability of Leu-Enk and DADLE in rat intestine and Caco-2 cell monolayers (18,19). Alternatively, chemical modification is one of the most promising approaches to improve the stability and permeability of enkephalins (20).

In this study, DADLE (Tyr-D-Ala-Gly-Phe-D-Leu), an enkephalin analogue, was chosen as a model peptide, and we synthesized new lipophilic derivatives of DADLE by chemical modification with various fatty acids at the C terminus to increase their stability and permeability in the intestine. We investigated the pharmacological activities of these DADLE derivatives and their stability and permeability by *in vitro* stability and transport studies using rat intestine. Furthermore, effects of protease inhibitors on the permeability of new lipophilic derivatives of DADLE were also investigated using rat intestinal membranes.

MATERIALS AND METHODS

Materials

DADLE was purchased from the Peptide Institute (Osaka, Japan). NaGC, amastatin, puromycin, and naltrindole were purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid, butyric acid, caproic acid, caprylic acid, trifluoroacetic acid, acetonitrile, and dimethylformamide were purchased from Nacalai Tesque (Kyoto, Japan). 5(6)-Carboxyfluorescein (CF) was kindly supplied by Eastman Kodak Co. (Rochester, NY). The acyl derivatives of DADLE

[1] Department of Biopharmaceutics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8414, Japan.

[2] Department of Medicinal Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8414, Japan.

[3] To whom correspondence should be addressed. (e-mail: yamamoto@mb.kyoto-phu.ac.jp)

were chemically synthesized as described below. All other chemicals were of the highest reagent grade available and were used without further purification.

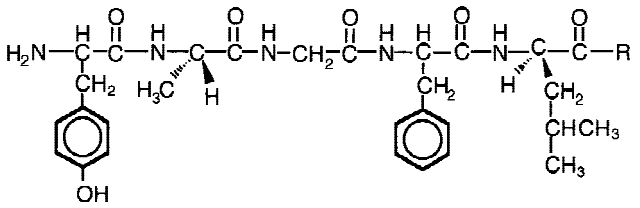
Synthesis of DADLE Derivatives

In this study, 4 DADLE (Tyr-D-Ala-Gly-Phe-D-Leu) derivatives were synthesized by chemical modification with various fatty acids according to the method of Barlos *et al.* (21). We synthesized C-terminal modified DADLE derivatives, since it was reported that the conformation of the hydroxyl group of Tyr at the N terminal was important for the activity of enkephalin via opioid δ receptors (22). Briefly, Boc-Tyr-D-Ala-Gly-Phe-D-Leu-OH was first synthesized using 2-chlorotrityl resin, and this N-terminal protected peptide was coupled with various fatty acids using 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide in the presence of 4-oxo-1,2,3,-benzotriazide (HOOBt). Then, the Boc group at the N-terminus was removed, and we obtained acetyl-DADLE (DADLE-C2), butyryl-DADLE(DADLE-C4), caproyl-DADLE(DADLE-C6), and caprylyl-DADLE (DADLE-C8). These new lipophilic derivatives of DADLE were identified by mass spectrometry. The chemical structures of these compounds are shown in Table I.

Determination of Lipophilicity of DADLE Derivatives

The lipophilicity of DADLE derivatives was determined by reversed phase high performance liquid chromatography (HPLC) (Shimadzu, Ltd., Kyoto, Japan) using a Cosmosil C18 column (4.6 × 150 mm). The column was eluted with various concentrations of methanol under isocratic conditions. The eluate was monitored with an ultraviolet detector at a wavelength of 214 nm. The lipophilic index values of chemically modified DADLE derivatives were calculated using the following equation: lipophilic index (log k') = log (T_R – T_0) / T_0, where T_R is the retention time of chemically modified DADLE derivatives, and T_0 is that of the solvent. For all the tested compounds, plots of log k' versus methanol concentration (v/v %) from 30% to 70% showed a reasonable linear relationship. The log k' values extrapolated to 0% methanol concentration are listed in Table I.

Table 1. Chemical Structure and Physicochemical Properties of DADLE and Its Derivatives



	R	M.W.	log k′[1]
DADLE	- OH	569.7	0.77
DADLE-C2	- NHNH-C(=O)-CH$_3$	625.8	0.83
DADLE-C4	- NHNH-C(=O)-(CH$_2$)$_2$-CH$_3$	653.8	0.88
DADLE-C6	- NHNH-C(=O)-(CH$_2$)$_4$-CH$_3$	681.9	0.93
DADLE-C8	- NHNH-C(=O)-(CH$_2$)$_6$-CH$_3$	709.9	0.98

[1] log *k′;* lipophilic index.

Determination of Pharmacological Activities of DADLE Derivatives

The hot plate test was used to evaluate the analgesic effects of DADLE and its derivatives (23). The test was performed on ddY male albino mice, with body weights of 25–35g. The test was carried out with the accessors blinded to the analogue they were testing. By this method, the reaction times of mice placed on a copper plate heated to a mean (± range) temperature of 55 ± 0.2°C were determined. Mice were first tested to determine a control latency (in seconds) in displaying hindpaw licking or jumping behavior. Those animals with control latencies of 20 sec or less were used for drug testing. Response latencies were again determined at predetermined times after intraperitoneal administration of DADLE (30 mg/kg) or its derivatives (equivalent to 30 mg/kg DADLE) in saline solution. Mice were intraperitoneally injected with a δ receptor-selective antagonist naltrindole (5 mg/kg) and was intraperitoneally injected 30 min later with DADLE or a derivative. The doses of these compounds were selected according to a previous report (23). A maximal test latency of 30 sec was used to avoid tissue damage. From these latencies, the percentage of analgesia was calculated as follows:

$$\text{Percentage analgesia} = (\text{test latency} - \text{control latency}) \times 100 / (30 - \text{control latency}).$$

Absorption Experiments

Absorption experiments were performed in a modified Ussing chamber (surface area, 0.3 cm^2) using stripped rat intestine at 37°C for 2 h (24). Male Wistar rats, weighing 200–250 g, were used. The studies performed in this paper were carried out in accordance with the guidelines of the animal ethics committee at Kyoto Pharmaceutical University. The intestine was excised and rinsed in phosphate buffer solution. The experimental segments were obtained, and the underlying muscularis was removed before being mounted in a modified Ussing chamber. The first 10 cm portion of the small intestine from the stomach (duodenum) was removed and the second 10 cm portion of the small intestine was used as the jejunum. Similarly, the first 10 cm portion of the large intestine from the ileo-cecal junction was removed and the second 10 cm of the large intestine was used as the colon. 2.5 ml of modified Ringer's solution was added to the serosal side. An equal volume of drug solution was added to the mucosal side. Mixing was performed by bubbling with 95% O_2 – 5% CO_2 gas. At pre-determined times, 200 μl aliquots were taken from the serosal side and the permeated DADLE or derivatives were assayed by UV detection HPLC. The apparent permeability coefficients (Papp) were calculated by the function: Papp = $dX_R/dT \cdot 1/A \cdot C_0$, where Papp is the apparent permeability coefficient in centimeters per second, X_R is the amount of drug in moles on the receptor side, A is the diffusion area (i.e., in square centimeters) and C_0 is the initial concentration of drugs on the donor side in moles per milliliter. The metabolites of DADLE and its derivatives were not detected on the receptor side during the transport studies. In

certain experiments, 0.5 mM amastatin, puromycin, or NaGC was added to the drug solution. The concentration (0.5 mM) of these additives was pre-determined, because this concentration was effective in improving the stability and permeability of leucine-enkephalin and DADLE as described previously (18). The viability of the intestinal membranes during the test period was monitored by measuring the transport of trypan blue dye and electrophysiological parameters including potential difference (PD), short circuit current (Isc) and membrane resistance (Rm). Under the initial conditions, PD, Isc, and Rm were approximately 2.9 mV, 18.0 μA, and 124.3 $\Omega \cdot cm^2$, respectively. There was no transport of dye during the incubation and no marked change on the electrophysiological parameters, confirming that the viability of the intestinal membrane was maintained during the transport experiment.

Preparation of Tissue Homogenates and Plasma

Intestinal mucosal tissue homogenates were prepared according to the method of Yamamoto *et al.* with slight modifications (25). Briefly, male Wistar rats, weighing 200–250g, were anesthetized with sodium pentobarbital (32 mg/kg body weight, i.p.). Animals fasted for about 16 h prior to the experiments, but were allowed water *ad libitum.* After washing the luminal surface with a saline solution, jejunal and colonic mucosae were removed by scraping the epithelial cell layers. These specimens were homogenized in a Polytron homogenizer (Kinematica AG, Switzerland). The liver homogenate was prepared by a similar method after the infusion of 0.15M NaCl (saline) into the portal vein to avoid blood contamination. Each homogenate was centrifuged at 3000 × g in a refrigerated (4°C) centrifuge for 10 min to remove cellular and nuclear debris. The resulting supernatant was adjusted with phosphate buffered saline (PBS) to a protein concentration of 5 mg/ml, as determined by the method of Lowry *et al.* with bovine serum albumin as the standard (26). Plasma was also adjusted with PBS to the same protein concentration as the tissue supernatant.

Degradation of DADLE and Its Derivatives in the Various Homogenates

The degradation of DADLE and modified DADLE derivatives was studied by incubating 300 μl of tissue supernatant, which had been preincubated at 37°C for 15 min, and 300 μl of 0.3 mM DADLE or chemically modified DADLE solution. At predetermined times up to a maximum of 120 min, 50 μl aliquot were sampled and 100 μl of 50% acetic acid was added to terminate the reaction. The resulting mixture was centrifuged at 10,000 rpm for 5 min to remove the precipitated protein. These samples were analyzed by HPLC. The degradation of these compounds followed first order kinetics. Therefore, the degradation rate constant and half-life were calculated from the drug percentage remaining according to the incubation solution-time profiles from the stability studies.

Analytical Methods

Aliquots were assayed in a reversed-phase HPLC system containing 5 μM Cosmosil (4.6 mm × 15 cm) particles in an analytical column from Nacalai Tesque, a Shimadzu LC-10 pump system, a Shimadzu LC-10 autoinjector, a Shimadzu LC-10 detector, and a Shimadzu CR-6A integrator. The mobile phase was a binary mixture of varying proportions of acetonitrile and water containing 0.1% H_3PO_4 and 0.1 M $NaClO_4$. The amount of acetonitrile in the mobile phase was increased linearly from 5 to 17% over the first 12 min and from 17 to 50% over the next 20 min at a flow rate of 1.0 ml/min. DADLE and its derivatives were monitored spectrophotometrically at 214 nm. Concentrations were determined using external standards.

Statistical Analyses

Results were expressed as the mean ± S.E. and statistical significance was assessed by the Student's t-test with $p < 0.05$ as the minimal level of significance.

RESULTS

Physicochemical Properties and Pharmacological Activity of DADLE Derivatives

The physicochemical properties and pharmacological activities of DADLE derivatives are shown in Table I. The molecular weight and lipophilicity of DADLE were increased with increasing acyl chain length attached to native DADLE. However, the melting point of chemically modified DADLE was similar to that of DADLE (data not shown). The pharmacological activities of chemically modified DADLE gradually decreased with increasing acyl chain length, as shown in Fig. 1. However, all the derivatives of DADLE had at least 75% of the activity of the native compound. Of these derivatives, DADLE-C2 and DADLE-C4 had almost the same activities as DADLE. Therefore, acyl modification at the C terminus did not markedly affect the pharmacological activity of DADLE. In contrast, our preliminary study demonstrated that a significant decrease in pharmacological activity was observed in the case of N terminal modifications, C4-DADLE, and C6-DADLE (data not shown). To evaluate whether the analgesic activity was mediated via a δ receptor, we examined the analgesic activities of DADLE and its derivative, DADLE-C4 in the presence of naltrindole, a specific δ receptor antagonist. In this study, naltrindole was given prior to intraperitoneal administration of DADLE or its derivatives. The analgesic effects of DADLE and DADLE-C4 were antagonized and the percent analgesia was almost the same as the control level.

The Stability of DADLE and Its Derivatives in Various Homogenates

Table II shows the half-lives of the hydrolysis of DADLE and its derivatives in plasma and in various homogenates. The decreasing order of half lives was colon > jejunum > liver > plasma. In the homogenates of jejunal and colonic mucosae, the stability of DADLE increased as the acyl chain length increased. However, chemical modification did not improve the stability of DADLE in plasma and liver homogenates. Furthermore, DADLE and its derivatives were not degraded in intestinal mucosal fluid (data not shown).

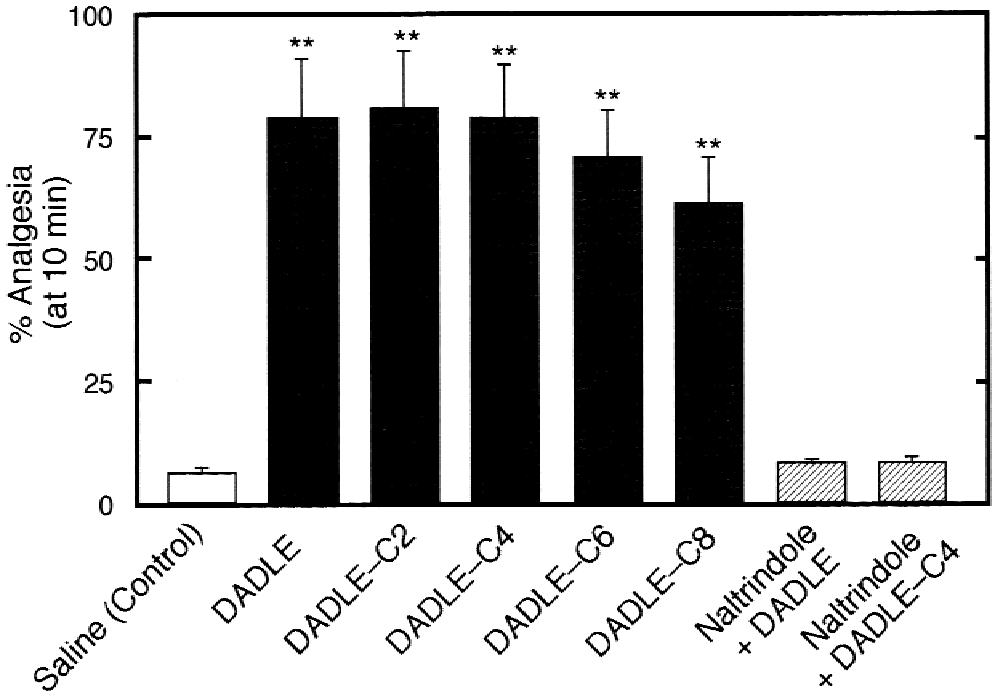


Fig. 1. Analgesic activities of DADLE derivatives and the effects of naltrindole, a specific δ-receptor antagonist, on the analgesic activities of DADLE derivatives determined by a hot-plate antinociceptive test after intraperitoneal administration. Percentage analgesia = (test latency – control latency) × 100 / (30 – control latency). Each point represents the mean ± S.E. of at least four experiments. (**) $p < 0.01$, compared with the saline (control).

Table 2. Half-lives for the Hydrolysis of DADLE and Its Derivatives (0.3 mM) in Plasma, Liver Homogenate, and Homogenates of Jejunal and Colonic Mucosae in Rats

	$T_{1/2}$ (min)			
	Plasma	Liver	Jejunum	Colon
DADLE	17.8 ± 2.4	57.9 ± 4.9	126 ± 3.2	240 ± 5.8
DADLE-C2	18.4 ± 2.5	58.6 ± 5.1	103 ± 2.1**	199 ± 4.9**
DADLE-C4	18.2 ± 3.1	56.2 ± 4.5	142 ± 3.9*	250 ± 6.1
DADLE-C6	18.1 ± 2.7	57.8 ± 5.3	160 ± 3.7**	268 ± 6.2*
DADLE-C8	18.2 ± 2.5	59.1 ± 4.8	176 ± 4.1**	298 ± 6.3**

Note: Each value represents the mean ± S.E. of three experiments. (*) $p < 0.05$, (**) $p < 0.01$, compared with DADLE.

Permeability of DADLE and Its Derivatives Across the Jejunal and Colonic Membranes

Fig. 2 shows permeation profiles of DADLE and its derivatives across the rat jejunal and colonic membranes. The permeability of DADLE derivatives was higher than native DADLE except for DADLE-C8. In particular, the permebility of DADLE-C2 and DADLE-C4 was much greater than native DADLE. In contrast, the Papp value of DADLE-C8 was lower than native DADLE. Fig. 3 represents the relationship between Papp of DADLE and its derivatives and lipophilicity. In both jejunal and colonic membranes, a "bell-shaped" profile was seen in the relationship between these two parameters. Thus, there appears to be an optimal lipophilicity for the intestinal permeability of DADLE deriva-

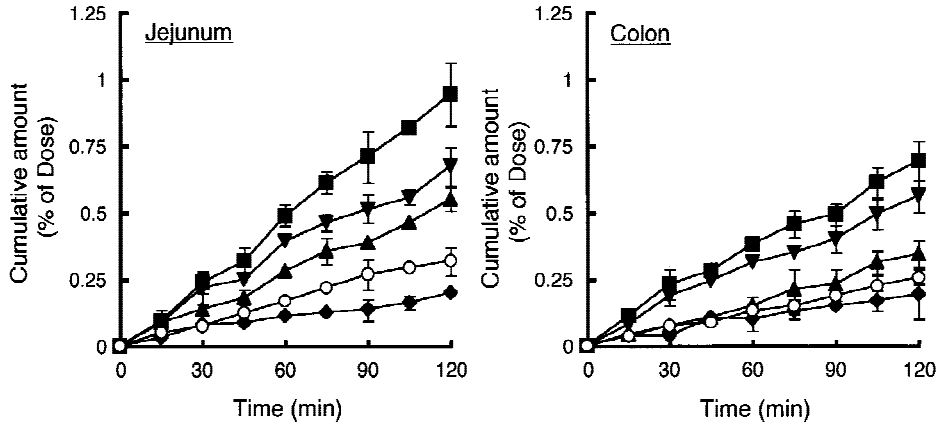


Fig. 2. Permeation profiles of DADLE and its derivatives across jejunal and colonic membranes. Results are expressed as the mean ± S.E. of at least three experiments. Key: DADLE (○), DADLE-C2 (▼), DADLE-C4 (■), DADLE-C6 (▲), DADLE-C8 (♦).

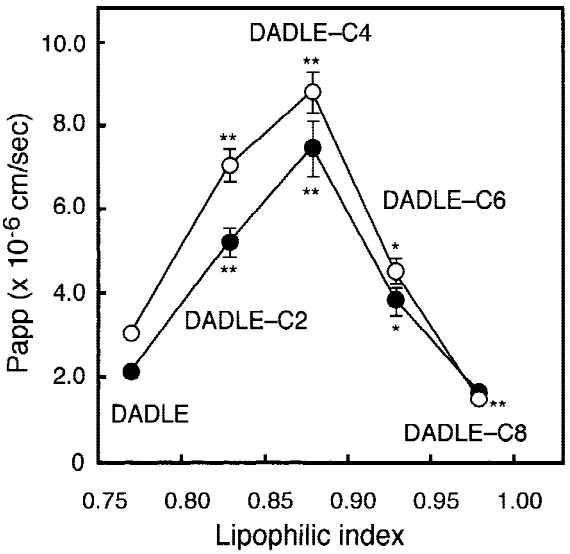


Fig. 3. Relationship between the apparent permeability coefficients of DADLE derivatives and lipophilicity. Key: jejunum (○), colon (●). Each point represents the mean ± S.E. of at least three experiments. (*) $p < 0.05$, (**) $p < 0.01$, compared with DADLE.

tives. The permeability of DADLE-C8 was lowest despite its high lipophilicity.

Effects of DADLE Derivatives on the Permeability of CF Across the Jejunal and Colonic Membranes

It may be possible that DADLE derivatives have absorption-enhancing effects due to their acyl moiety. Therefore, CF, a water soluble and stable compound, was selected as a model compound and the absorption enhancing effects of the acyl moiety of DADLE-C4 were investigated. In jejunum, the Papp value of CF (0.1 mM) without DADLE-C4 (1 mM) was 4.85 ± 0.38 ($\times 10^{-6}$ cm/sec) and it was 4.88 ± 0.34 ($\times 10^{6}$ cm/sec) in the presence of DADLE-C4. Similarly, the Papp of CF across the colonic membrane in the presence or absence of DADLE-C4 was 3.19 ± 0.30 ($\times 10^{-6}$ cm/sec) or 3.22 ± 0.28 ($\times 10^{-6}$ cm/sec), respectively. Therefore, DADLE-C4 did not increase the permeability of CF across the jejunal and colonic membranes, suggesting that the acyl moiety of DADLE-C4 had no absorption enhancing effect.

Effects of Various Protease Inhibitors on the Permeability of New DADLE Derivatives Across the Jejunal and Colonic Membranes

Finally, we examined the synergistic effects of chemical modification and various protease inhibitors for improving the permeability of DADLE across the jejunal and colonic membranes. As shown in Fig. 4, the permeability of DADLE-C4 across the jejunal membrane was further improved in the presence of puromycin, amastatin, and NaGC all at a concentration of 0.5 mM, whereas these protease inhibitors except for NaGC were not effective in enhancing the colonic permeability of DADLE-C4. The combination effect was more marked in the jejunum that in the colon. Of these protease inhibitors, NaGC most effectively improved the permeability of DADLE-C4 across the jejunal and colonic membranes.

DISCUSSION

In this study, we synthesized novel lipophilic derivatives of DADLE by chemical modification with various fatty acids at the C terminus. We found that all the derivatives had at least 75% of the activity of native DADLE. In contrast, we observed a significant decrease in the pharmacological activities of DADLE derivatives modified with butyric acid (C4-DADLE) and caproic acid (C6-DADLE) at the N terminus in our pilot studies. It was reported that the important positions for maintaining the pharmacological activity of DADLE are Tyr at the N terminus, Gly in position 3, an aromatic ring of Phe in position 4, and the distances between the aromatic ring of Phe in position 4 and Tyr at the N terminus (27). These findings indicated that chemical modification of DADLE at the C terminus is preferable in terms of maintaining its pharmacological activity rather than at its N terminal.

In our previous studies, we indicated that the pharmaco-

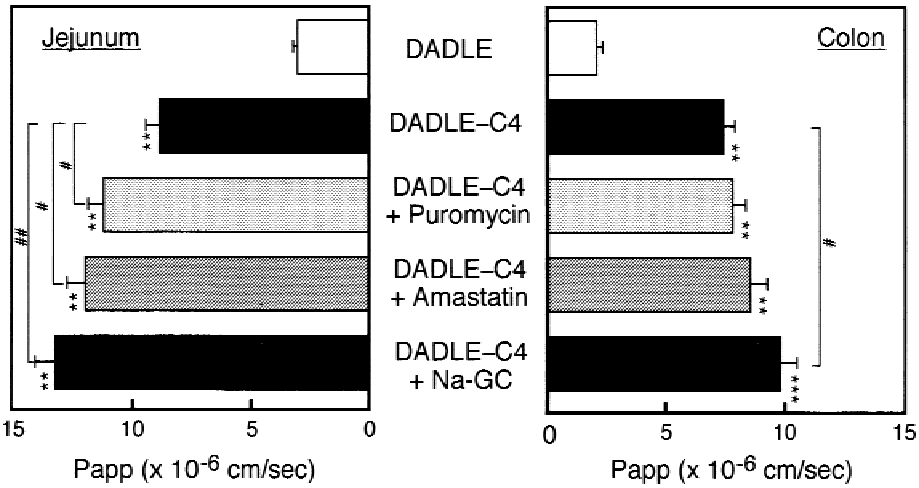


Fig. 4. Synergistic effects of acylation and protease inhibitors on the apparent permeability coefficients of DADLE in the jejunal and colonic membranes. Results are expressed as the mean ± S.E. of at least three experiments. All protease inhibitors were used at 0.5 mM. (**) $p < 0.01$, (***) $p < 0.001$, compared with DADLE. (#) $p < 0.05$, (##) $p < 0.01$, compared with DADLE-C4.

logical activities of peptide drugs such as insulin, calcitonin, tetragastrin and thyrotropin-releasing hormone (TRH) were not markedly affected by chemical modification with fatty acids (6, 7, 8, 12). Accordingly, these previous findings are in good agreement with our present findings on DADLE derivatives. However, the pharmacological activities of these DADLE derivatives were gradually reduced with increasing acyl chain length of the fatty acid attached to native DADLE. This finding concurs with the previous report for acyl–insulin derivatives. Presumably, these reduced pharmacological activities of DADLE derivatives may be attributed to decreased receptor binding affinity due to steric hindrance. On the other hand, we found that the analgesic effects of DADLE and DADLE-C4 were completely antagonized by naltrindole, which is a specific δ receptor antagonist (28), and the percent analgesia was almost the same as the control level. These findings suggested that the analgesic activities of both DADLE and DADLE-C4 are mediated via a δ receptor.

Enkephalins are known to be easily degraded by aminopeptidases, carboxypeptidases and enkephalinases (17), while DADLE is relatively stable and is resistant to these proteases. This higher stability of DADLE may be due to the substitution of L-Gly to D-Ala at the second amino acid position and L-Leu to D-Leu at the C terminal amino acid. However, we found mild degradation of DADLE in the jejunal and colonic mucosal homogenates, although the degradation half-life of DADLE was much longer than Leu-Enk, as reported previously (18). This study demonstrated that there was no significant difference in stability between DADLE and its derivatives in the plasma and liver homogenates. This finding may be attributable to the larger amount of proteases in the liver and plasma, and that the stability of DADLE was not improved even after modification of the chemical structure with various fatty acids. In contrast, the stability of DADLE derivatives in the intestine was improved by increasing acyl chain length. The higher stability of these DADLE derivatives in the intestinal homogenates may be explained by the inhibition of carboxypeptidase, since this enzyme cleaves the peptide bond between Phe in position 4 and D-Leu in position 5 of native DADLE. However, the metabolites of DADLE (D-Ala-Gly-Phe-Leu and Gly-Phe-Leu) by aminopeptidase and dipeptidylaminopeptidase were detected by HPLC assay. Accordingly, the inhibition of these other peptidases may also be related to the metabolism of DADLE in the intestine.

In the transport studies, a "bell-shaped" profile was seen between the apparent permeability coefficients of the DADLE derivatives in the jejunum and colon and their lipophilicity. We observe higher permeabilities for DADLE-C2, DADLE-C4, and DADLE-C6 in comparison with native DADLE, and DADLE-C4 showed the highest permeability across the jejunum and colon. In contrast, the permeability of DADLE-C8 was lower than native DADLE despite being the most lipophilic derivative. The higher permeability of these derivatives in comparison with native DADLE might be accounted for by their lipophilicity. Enhanced intestinal absorption of peptides by acylation was also observed in the case of insulin (4–6), calcitonin (7), tetragastrin (8,9) and TRH (12,13). In particular, we found a similar "bell-shaped" profile between the permeability of acyl-tetragastrins across the intestinal membrane and lipophilicity (9). Therefore, our present findings concur with the previous findings on acyl-tetragastrin. On the other hand, the lack of permeability of DADLE-C8 may be attributed to its reduced diffusion in the cytosol because of its strong partition and binding to the brush border membranes of the intestinal epithelia. Furthermore, Lang *et al.* reported that the permeability of metkephamid, an enkephalin analogue, was direction-specific in Caco-2 cells, and that it might be secreted via a p-glycoprotein (P-gp)-mediated efflux mechanism (29). Therefore, it may be possible that P-gp having a broad substrate specificity might be related to this low apparent permeability characteristic of DADLE-C8, although we have no evidence to support this speculation at present.

It is known that fatty acids such as sodium caprate have the ability to enhance intestinal absorption of many drugs including peptide drugs (30). However, DADLE-C4 did not increase the transport of CF, a water-soluble compound, across the jejunal and colonic membranes, indicating that the acyl moiety of DADLE derivatives had no absorption enhancing effect. This finding is inconsistent with our previous findings on the absorption enhancing effects of acyl-tetragastrins across Caco-2 cell monolayers (7). The discrepancy is not clearly understood. One explanation for the discrepancy may be due to different experimental conditions. In the previous studies, we used Caco-2 cell monolayers as a model of intestine, while the present studies were performed using intact rat intestine. Caco-2 cells may be more sensitive to fatty acids than intact rat intestine at the same concentration.

This study demonstrated that the permeability of DADLE-C4 across the jejunal membrane was further improved in the presence of puromycin, amastatin, and NaGC all at a concentration of 0.5 mM. Previously, we reported that the stability of DADLE in the intestinal homogenate was improved by the addition of various aminopeptidase inhibitors such as bestatin, puromycin, amastatin, and NaGC (18). Therefore, the increased permeability of DADLE-C4 by various protease inhibitors may be partly explained by the enhanced stability of DADLE-C4 due to inhibition of aminopeptidase activities. Alternatively, we may consider the contribution of absorption enhancement of NaGC, since bile salts are generally known to be typical absorption enhancers (1). However, it was reported that amastatin and puromycin did not have absorption-enhancing properties (18). Therefore, these protease inhibitors, except for NaGC, enhanced the permeability of DADLE-C6 via their inhibitory activities rather than their absorption-enhancing actions. Moreover, the synergistic absorption-enhancing effects of chemical modification and protease inhibitors support our previous finding that intestinal absorption of calcitonin was enhanced by the combination effects of acyl-modification and the application of protease inhibitors (7). Therefore, we suggest that the combination of chemical modification and protease inhibitors is an effective method to improve the absorption of peptide drugs across the intestinal membrane.

In summary, we demonstrated that the intestinal permeability of novel lipophilic DADLE derivatives, except for DADLE-C8, was higher than native DADLE. In addition, we indicated that protease inhibitors further improved the absorption of DADLE-C4, a new DADLE derivative, across the intestinal membrane. Therefore, a combination of chemical modification with butyric acid and the application of a protease inhibitor are effective for improving the intestinal absorption of DADLE.

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REFERENCES

1. V. H. L. Lee and A. Yamamoto. Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Delivery Rev.* **4**:171–207 (1990).
2. A. Yamamoto and S. Muranishi. Improvement of rectal peptide absorption by absorption enhancers, protease inhibitors and chemical modification. *Adv. Drug Delivery Rev.* **28**:275–299 (1997).
3. H. Bundgaard. (C) Means to enhance penetration. (1) Prodrugs as a means to improve the delivery of peptide drugs. *Adv. Drug Delivery Rev.* **8**:1–38 (1992).
4. M. Hashizume, T. Douen, M. Murakami, A. Yamamoto, K. Takada, and S. Muranishi. Improvement of large intestinal absorption of insulin by chemical modification with palmitic acid in rats. *J. Pharm. Pharmacol.* **44**:555–559 (1992).
5. H. Asada, T. Douen, Y. Mizokoshi, T. Fujita, M. Murakami, A. Yamamoto, and S. Muranishi. Stability of acyl derivatives of insulin in the small intestine: Relative importance of insulin association characteristics in aqueous solution. *Pharm. Res.,* **11**:1115–1120 (1994).
6. H. Asada, T. Douen, M. Waki, S. Adachi, T. Fujita, A. Yamamoto, and S. Muranishi. Absorption characteristics of chemically modified-insulin derivatives with various fatty acids in the small and large intestine. *J. Pharm. Sci.* **84**:682–687 (1995).
7. T. Fujita, T. Fujita, K. Morikawa, H. Tanaka, O. Iemura, A. Yamamoto, and S. Muranishi. Improvement of intestinal absorption of human calcitonin by chemical modification with fatty acids: Synergistic effects of acylation and absorption enhancers. *Int. J. Pharm.* **134**:47–57 (1996).
8. T. Tenma, E. Yodoya, S. Tashima, T. Fujita, M. Murakami, A. Yamamoto, and S. Muranishi. Development of new lipophilic derivatives of tetragastrin: Physicochemical characteristics and intestinal absorption of acyl-tetragastrin derivatives in rats. *Pharm. Res.* **10**:1488–1492 (1993).
9. E. Yodoya, K. Uemura, T. Tenma, T. Fujita, M. Murakami, A. Yamamoto, and S. Muranishi. Enhanced permeability of tetragastrin across the rat intestinal membrane and its reduced degradation by acylation with various fatty acids. *J. Pharmacol. Exp. Ther.* **271**:1509–1513 (1994).
10. K. Setoh, M. Murakami, N. Araki, T. Fujita, A. Yamamoto, and S. Muranishi. Improvement of transdermal delivery of tetragastrin by lipophilic modification with fatty acids. *J. Pharm. Pharmacol.* **47**:808–811 (1995).
11. T. Fujita, I. Kawahara, Y-S. Quan, K. Hattori, K. Takenaka, S. Muranishi, and A. Yamamoto. Permeability characteristics of tetragastrins across intestinal membranes using the Caco-2 monolayer system: Comparison between acylation and application of protease inhibitors. *Pharm. Res.* **15**:1387–1392 (1998).
12. K. Yamada, M. Murakami, A. Yamamoto, K. Takada, and S. Muranishi. Improvement of intestinal absorption of thyrotropin-releasing hormone by chemical modification with lauric acid. *J. Pharm. Pharmacol.* **44**:717–721 (1992).
13. K. Tanaka, T. Fujita, Y. Yamamoto, M. Murakami, A. Yamamoto, and S. Muranishi. Enhancement of intestinal transport of thyrotropin-releasing hormone via a carrier-mediated transport system by chemical modification with lauric acid. *Biochim. Biophys. Acta* **1283**:119–126 (1996).
14. T. Fujita, Y. Morishita, H. Ito, D. Kuribayashi, A. Yamamoto, and S. Muranishi. Enhancement of the small intestinal uptake of phenylalanyl-glycine via a H^+/oligopeptide transport system by chemical modification with fatty acids. *Life Sci.* **61**:2455–2465 (1997).
15. S. Dodda-Kassi and V. H. L. Lee. Enkephalin hydrolysis in homogenates of various absorptive mucosae of the albino rabbit: similarities in rates and involvement of aminopeptidases. *Life Sci.* **38**:2019–2028 (1986).
16. G. A. Kerchner and L. E. Geary. Studies on the transport of enkephalin-like oligopeptides in rat intestinal mucosa. *J. Pharmacol. Exp. Ther.* **226**:33–38 (1983).
17. D. I. Friedman and G. L. Amidon. Oral absorption of peptides: Influence of pH and inhibitors on the intestinal hydrolysis of leu-enkephalin and analogues. *Pharm. Res.* **8**:93–96 (1991).
18. T. Uchiyama, A. Kotani, T. Kishida, H. Tatsumi, A. Okamoto, T. Fujita, M. Murakami, S. Muranishi, and A. Yamamoto. Effects of various protease inhibitors on the stability and permeability of (D-Ala^2, D-Leu^5) enkephalin in the rat intestine: Comparison with leucine enkephalin. *J. Pharm. Sci.* **87**:448–452 (1998).
19. Y-S. Quan, T. Fujita, D. Tohara, M. Tsuji, M. Kohyama, and A. Yamamoto. Transport kinetics of leucine enkephalin across Caco-2 monolayers: Quantitative analysis for contribution of enzymatic and transport barrier. *Life Sci.* **64**:1243–1252 (1999).
20. T. Mizuma, K. Ohta, A. Koyanagi, and S. Awazu. Improvement of intestinal absorption of leucine enkephalin by sugar coupling and peptidase inhibitors. *J. Pharm. Sci.* **85**:854–857 (1996).
21. B. Barlos, D. Gatos, J. Kapolos, G. Papaphotiu, W. Schafer, and Y. Wenqing. Veresterung von partiell geschutzten peptidfragmenten mit harzen. Einsatz von 2-chlortritylchlorid zur synthese von Leu^{15}-gastrin I. *Tetrahedron Lett.* **30**:3947–3950 (1989).
22. M. Goodman and K. C. Stueben. Peptide syntheses via amino acid active esters. *J. Am. Chem. Soc.* **81**:3980–3983 (1959).
23. M. B. Tyers. A classification of opiate receptors that mediate antinociception in animals. *Br. J. Pharmacol.* **69**:503–512 (1980).
24. A. Yamamoto, E. Hayakawa, Y. Kato, A. Nishiura, and V. H. L. Lee. A mechanistic study on enhancement of rectal permeability to insulin in the albino rabbit. *J. Pharmacol. Exp. Ther.* **263**:25–31 (1992).
25. A. Yamamoto, E. Hayakawa, and V. H. L. Lee. Insulin and proinsulin proteolysis in mucosal homogenates of the albino rabbit: Implications in peptide delivery from nonoral routes. *Life Sci.* **47**:2465–2474 (1990).
26. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
27. Y. Kiso, M. Yamaguchi, T. Akita, H. Moritoki, M. Takei, and H. Nakamura. Super-active enkephalin analogs. Simple tripeptide hydroxyalkylamides exhibit surprisingly high and long-lasting opioid activities. *Naturwissenchaften* **68**:S210–S212 (1981).
28. P. S. Portoghese, M. Sultana, H. Nagase, and A. E. Takemori. Application of the message-address concept in the design of highly potent and selective non-peptide δ opioid receptor antagonists. *J. Med. Chem.* **31**:281–282 (1988).
29. V. B. Lang, P. Langguth, C. Ottiger, H. Wunderli-Allenspach, D. Rognan, B. Rothen-Rutishauser, J-C. Perriard, S. Lang, J. Biber, and H. P. Merkle. Structure-permeation relations of met-enkephalin peptide analogues on absorption and secretion mechanisms in Caco-2 monolayers. *J. Pharm. Sci.* **86**:846–853 (1997).
30. T. Uchiyama, T. Sugiyama, Y-S. Quan, A. Kotani, N. Okada, T. Fujita, S. Muranishi, and A. Yamamoto. Enhanced permeability of insulin across the rat intestinal membrane by various absorption enhancers: Their intestinal mucosal toxicity and absorption-enhancing mechanism of n-lauryl-β-D-maltopyranoside. *J. Pharm. Pharmacol.* **51**:1241–1250 (1999).